AsA/DHA Redox Pair Influencing Plant Growth and Stress Tolerance

Javier Alberto Miret and Maren Müller

Abstract In a continuously changing environment plants are exposed to adverse stress conditions, such as sunlight, drying, cold, salinity, pollution, or heavy metals, which influence plant growth and result in the generation of reactive oxygen species (ROS). These small and highly reactive molecules have important cell signalling information concerning the change in the environmental and developmental conditions when maintained at proper cellular concentrations. However, during stress conditions, ROS levels in cells can greatly increase and cause oxidative stress by modifying other reactive species, proteins, or lipids. Therefore, appropriate regulation of ROS has a significant impact on plant development, growth, and survival. Ascorbic acid (AsA) as a major antioxidant in plant cells and its oxidized form dehydroascorbate (DHA) play a key role in redox state-based signalling mechanisms by detoxification of ROS and its products, as well as transmission of redox signals. Furthermore, DHA by itself also presents unique functions: cell cycle progression sensing and regulation, modulation of metal stress responses, and DHA adducts seem to be involved in oxidative stress-mediated cellular toxicity. It has become clear that the changes in the pool and ratio of the AsA/DHA redox pair by both growth and environmental cues modulate gene expression and protein levels resulting in increased stress tolerance. In the recent years, this important redox couple (AsA/DHA) has been of increasing interest to better understand the mechanisms of adaptive plant responses and stress tolerance towards abiotic and biotic stress. In this chapter, an overview of the literature is briefly presented in terms of the role of AsA/DHA redox pair in plant growth, and abiotic and biotic stress tolerance.

Keywords Ascorbic acid · Dehydroascorbate · Redox state · Plant growth · Abiotic stress · Biotic stress · Reactive oxygen species

J. A. Miret · M. Müller (✉)
Department of Evolutionary Biology, Ecology and Environmental Sciences, Plant Physiology Section, Faculty of Biology, University of Barcelona, Barcelona, Spain
e-mail: maren.muller@ub.edu

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1 Introduction

The introduction of molecular oxygen to our early reducing atmosphere led to O_2-evolving photosynthetic active organisms in the evolution of aerobic life and, as a consequence, to the formation of reactive oxygen species (ROS). Initially, ROS were recognized as toxic by-products generated constantly by aerobic metabolism. However, recently it has become apparent that ROS also play important signalling roles, controlling processes such as growth, development, and especially responses to abiotic and biotic stresses. The major members of the ROS family include free radicals like superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$) and non-radicals like hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Mittler 2002). During the life cycle of plant cells, ROS are formed under normal and stressful conditions in the different cellular compartments. The major generators are chloroplasts, mitochondria, and peroxisomes; but other compartments also produce relevant amounts of ROS: plasma membrane, endoplasmic reticulum, and apoplast (Jubany-Marí et al. 2009; Roychoudhury and Basu 2012). In the presence of light, chloroplasts and peroxisomes are the major sources of ROS production during reactions that participate in the mechanism of photosynthesis and photorespiration, respectively. Meanwhile, mitochondria are the leading producer of ROS during respiration under dark conditions (Choudhury et al. 2013; Mittler et al. 2004).

Under stress-free conditions, ROS, especially $O_2^{\bullet -}$ and H_2O_2, are constantly generated at rather low basal level acting as second messengers and key regulators of growth and plant development. These molecules, although being toxic to the cell, are unable to cause cellular damage as they are scavenged and tightly controlled by a complex antioxidant machinery (Foyer and Noctor 2005a; Mittler et al. 2004). However, under different kinds of environmental stresses, such as high light, high or low temperatures, salinity, drought, nutrient deficiency, and pathogen attack, the cellular homeostasis is disrupted; and this imbalance between ROS production and their detoxification causes oxidative stress (Mittler 2002; Chalapathi and Reddy 2008). ROS are oxidizing agents that are able to subtract electrons from essential organic molecules and thus disturb the cellular function of proteins, nucleic acids, lipids, and sugars, which may lead to cell damage and ultimately cell death. Therefore, the survival of stressed plants, as sessile organisms, depends on adaptation and avoidance strategies like change in growth conditions, severity and duration of stress conditions, and the capacity to quickly adapt a battery of antioxidant strategies (Miller et al. 2010; Foyer and Shigeoka 2011).

The antioxidant defense machinery includes: (1) enzymatic molecules like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), and dehydroascorbate reductase (DHAR) and (2) nonenzymatic antioxidants like AsA, reduced glutathione (GSH), α-tocopherol, carotenoids, flavonoids, and the osmolyte proline (Nobuhiro and Mittler 2006). The latter are low molecular weight antioxidants that facilitate the cell to be detoxified during extreme environmental stress conditions, and also to keep ROS at the optimum level allowing

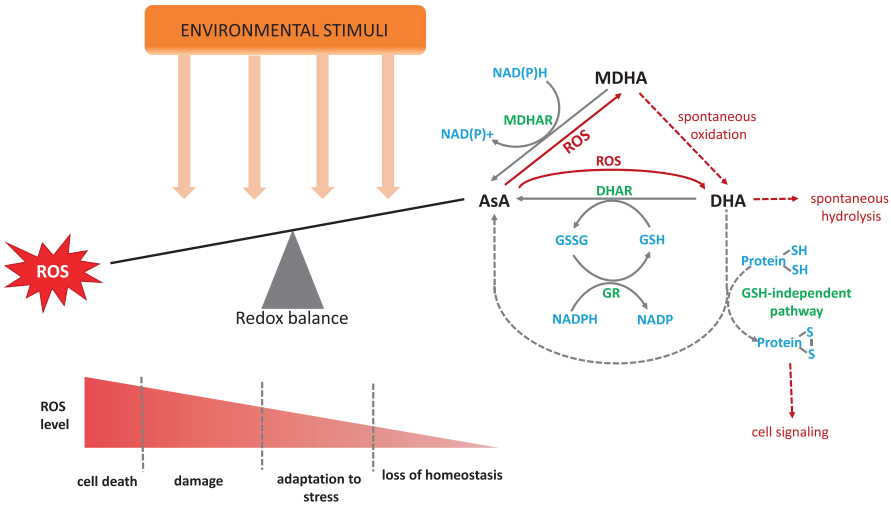

Fig. 1 Schematic representation of the oxidoreduction function of AsA/DHA redox pair during ROS detoxification. Optimum level of ROS keeps cell in homoeostasis condition for normal growth and development of plants. In contrast, different kinds of environmental stimuli trigger enhanced ROS formation, which disrupt cellular homoeostasis and may lead to cell damage or cell death. The survival of stress plants depends in part of quickly ROS detoxification by AsA/DHA redox pair. The AsA recycling involves DHA reduction, which includes (**a**) the AsA-GSH cycle and (**b**) a GSH-independent pathway has been proposed. *AsA* ascorbic acid, *DHA* dehydroascorbate, *DHAR* dehydroascorbate reductase, *GR* glutathione reductase, *GSH* reduced glutathione, *GSSG* oxidized glutathione, *MDHA* monodehydroascorbate, *MDHAR* monodehydroascorbate reductase, *ROS* reactive oxygen species

redox-sensitive signal transduction and balance information from environment and developmental stimuli (Foyer and Noctor 2005b).

AsA is the primary water-soluble antioxidant and has been detected in a wide range of cellular compartments like cytosol, chloroplast, vacuoles, mitochondria, and apoplast. The molecule is considered to be as one of the most powerful antioxidants in plant cells by a virtue of its ultra-crucial ability to function as a donor of electrons by delocalizing electrons around a 5-carbon ring. Due to its oxidoreduction potential, AsA interacts with hydroxyl radicals, singlet oxygen, superoxide, and also with glutathione and tocopherol radicals (Noctor and Foyer 1998). AsA can directly scavenge and neutralize ROS, but it can also repair oxidized molecules such as α-tocopherol or can serve as an enzyme cofactor, for example, for violaxanthin de-epoxidase (VDE) (Blokhina et al. 2003; Müller-Moulé et al. 2002). The regeneration system for AsA involves two key enzymes known as dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase (MDHAR) (Fig. 1). The primary oxidation product is the one-electron oxidized unstable radical monodehydroascorbate (MDHA). Two MDHA molecules can react each other to form one molecule of AsA or dehydroascorbate (DHA), which is the two-electron oxidized form of AsA. MDHAR uses NAD(P)H as an electron donor to reduce MDHA

before spontaneous oxidation to DHA. In the chloroplast and mitochondrion, the MDHA radical can also be reduced by ferredoxin and complex II, which is more effective than reduction by NAD(P)H-dependent MDHAR (Asada 1999; Szarka 2013). DHAR catalyzes the reduction of DHA to AsA using glutathione (GSH) as a hydrogen donor (ascorbate-glutathione cycle or Halliwell–Asada cycle). AsA recycling by DHAR, therefore, serves to recycle DHA into AsA before it is lost from the AsA pool. DHA, which predominates in the apoplastic space, must reenter the cell for reduction to AsA because the apoplast contains little GSH and DHAR amounts. In the absence of enough recycling activity, however, DHA is hydrolyzed to irreversible form 2,3-diketogulonic acid (Chen et al. 2003; Potters et al. 2002; Pignocchi and Foyer 2003; Munné-Bosch et al. 2013). Moreover, DHA seems to be able to directly interact not only with GSH, but also using an active GSH-independent pathway for DHA reduction (Fig. 1) (Potters et al. 2004; Fotopoulos et al. 2008).

In higher plants, the AsA/DHA redox pair functions as a reliable sensor that perceives and coordinates their action depending on the cellular redox state. Besides their action as reductant and reacting with and scavenging many types of ROS, AsA/DHA also influence many enzyme activities. Redox-sensitive proteins play crucial role in the ROS signal transduction, because they can undergo directly or indirectly reversible oxidation/reduction, which activate or deactivate them depending upon the cellular redox state. Whereas redox-sensitive metabolic enzymes can modulate directly appropriate cellular responses, redox-sensitive signalling proteins conduct their function via downstream signalling components including kinases, phosphatases, and transcription factors. Moreover, disulfide-thiol conversion is likely important in this redox signal transduction (Shao et al. 2008). It has been hypothesized that DHA could have a key role in signalling due to its peculiar reactivity with specific proteins, such as thiol-containing proteins (Potters et al. 2004; Fotopoulos et al. 2008). However, possible DHA-regulated target proteins are still to be identified in plants. In animal cells, for instance, protein-disulfide isomerase (PDI), a major protein of the ER lumen, is known to have DHA reductase activity. This enzyme accepts protein thiols as the source of reducing equivalents during protein thiol oxidation by DHA, while simultaneously AsA is formed (Bángegyi et al. 2003; Nardai et al. 2001).

Several studies conducted in a number of plant species under environmental stress conditions lead to the assumption that high AsA/DHA ratios accompanied by increasing AsA levels or decreasing DHA levels could be a key element coordinating efficient ROS protection (Szalai et al. 2009). Developmental and environmental stressors can rapidly challenge the AsA pool; however, changes in AsA biosynthetic capacity can take hours or longer and are tightly regulated by light and respiration electrons (Bartoli 2006). Although AsA and its redox state have a fundamental role in the plant defense system, they have also effects on many physiological processes including growth regulation, differentiation, and the metabolism of plants.

In this chapter, we present an overview of the literature that reveals aspects of the role of AsA/DHA redox pair in plant growth and development as well as tolerance responses to abiotic and biotic stresses.

2 The Role of AsA/DHA Redox Pair in Plant Growth and Development

The cellular redox state has emerged as an important determinant for plant growth and development due to the fact that the activity of genes and enzymes has specific redox requirements. Small changes of subcellular distribution of ROS, antioxidants, and redox state in different cell compartments can induce gene expression, defense signalling, and cell death (Kocsy et al. 2013). AsA and its redox state are the most important redox buffer for the detoxification of ROS in the apoplast and vacuoles. The cytosol acts as a hub for the AsA recycling as it reduces the oxidized forms produced in the cytosol or imported from other cell compartments. As AsA/DHA redox pair is also involved in protein synthesis and modification, it can be concluded that they act as oxidative stress sensors playing a key role in the fine-tuning of plant growth and development (Zechmann in press).

2.1 AsA and its Redox State Regulate Cell Cycle

Cell proliferation, the basis of plant growth, is under redox control. Low concentrations of ROS or shorter exposure of ROS seems to promote cell division in contrast to their excess or longer exposure, which can result to cell death. Concurrently, AsA and its redox state seem to play a role in the control of the cell cycle. An increase of AsA and MDHA promote cell division; whereas mutants of tobacco BY-2 cell lines, with 30% less AsA, showed a reduced cell division rate and growth (De Pinto et al. 1999; Kato and Esaka 1999). Moreover, increased level and activity of AsA/DHA redox pairs and ascorbate oxidase (AO) expression suggest that the oxidation of AsA seems to be crucial during cell elongation (Kato and Esaka 1999).

Within the quiescent center of onion root cells, AsA promotes cell division by inducing G1 to S progression (Liso et al. 1984), whereas exogenous DHA treatment reduced the mitotic activity of onion root meristems (De Cabo et al. 1993). In addition, exogenous addition of the plant hormone auxin in quiescent cells induced AO expression and activity, suggesting that high auxin levels induce AO expression, which seems to reduce AsA contents and maintain quiescent cells in G1 state of roots (Kerk and Feldmann 1995). In tobacco BY-2 cell cultures, the addition of DHA during G1 phase, but not during G2 phase, showed inhibitory effects on cell progression. Interestingly, exogenous DHA treatment only increased AsA levels, but not internal DHA concentrations, suggesting a rapid reduction of DHA to AsA. Increased AsA levels are associated with faster cell proliferation rates and not with inhibitory effects. Therefore the effect of DHA treatment may be, in part, by the depletion of GSH, as the latter is a cofactor during the reduction of DHA. Moreover, depletion of GSH seems to inhibit cell cycle progression (Potters et al. 2000, 2004). The reduction of DHA has also been proposed by a GSH-independent pathway and depletion of these reductants, such as thiol-containing

proteins, could also be involved in the inhibition of cell division by DHA treatment (Potters et al. 2004). Supporting this hypothesis is the observation that although AsA levels increased by exogenous L-galactono-1,4-lactone, the precursor to AsA, thiol-containing proteins were not oxidized (Paciolla et al. 2001). Given the effects of AsA and its redox state on the cell cycle, AsA promotes cell division whereas DHA seems to inhibit cell division. A hypothesis explained the impact of AsA and its redox state in stimulation of cell wall expansion and cellular growth (Smirnoff 1996). The model is based on the ability of AsA to give up electrons while the oxidized forms can accept electrons. Cytoplasmic AsA is oxidized by cytochrome b and electrons are transferred to MDHA in the apoplast. The transfer of electrons stimulates H^+-ATPase activity and leads to cell wall loosing stimulating cell growth. Moreover, AsA inhibits peroxidative cross-linking of cell wall polysaccharides and lignin polymerization by scavenging hydrogen peroxide radicals. Meanwhile, DHA may prevent cross-link to matrix polysaccharides and may react with amino acid side chain of cell wall glycoproteins. The AsA/DHA redox pair is also involved in protein synthesis and modification, which could contribute to the observed stimulatory effect of AsA on cell growth (Zechmann in press). The cell cycle progression could be controlled by an oxidative stress checkpoint pathway that responses to one or more redox-sensing systems, as has been proposed (Reichheld et al. 1999).

2.2 AsA and its Redox State Regulate Tissue and Organ Level

AsA and its redox state regulate not only at the cellular, but also at tissue and organ level growth and development. For example, seed development is characterized by dramatic changes of the AsA/DHA redox pair. First, levels of reduced AsA are high during early embryo development, followed by a decrease in the AsA redox state during cell elongation by the way that DHA levels exceed AsA levels (Tommasi et al. 2001). Orthodox seeds are characterized to withstand storage by a desiccation period at the end of the seed development, whereas recalcitrant seeds are desiccation sensitive seeds and are characterized by a short storage life. In orthodox seeds, when start to dry out at the end of seed development, AsA levels are completely oxidized in the embryo (Tommasi et al. 2001; Arrigoni et al. 1992); however, during germination DHA can be rapidly reduced to generate AsA (Tommasi et al. 2001). In contrast, recalcitrant seeds are able to germinate directly after seed abscission and remain metabolically very active. Therefore these seeds accumulate high levels of AsA/DHA redox pairs (Tommasi et al. 1999).

During embryo development, it has been observed that increasing endogenous AsA levels induce monozygotic twinning and polycotyly due to increasing DHAR expression in tobacco (Chen and Gallie 2012). Normally, early embryo development includes the transverse division of a zygote into an apical and a basal cell. However, the effect of AsA on monozygotic twinning generates two genetically identical zygotes, each of which develops into an independent embryo of equal size. The monozygotic twinning by AsA is limited to the first 2 days after pollination,

whereas polycotyly is induced when AsA levels increased just prior to cotyledon initiation. The frequency of polycotyly increases during cell division throughout the specification of cotyledon-forming.

Due to the fact that AsA/DHA redox pair is involved in cell division and elongation, their levels are correlated with leaf growth. However, AsA synthesis declines with the decrease in leaf function as a part of senescence and cell death process (Borraccino et al. 1994; Chen and Gallie 2006). Growth reduction could also be observed in tobacco plants with repressed DHAR expression resulting in lower recycling rate of AsA and its redox state (Chen and Gallie 2006). The *vtc1* mutants of *Arabidopsis*, deficient in AsA biosynthesis, showed significant growth reduction compared to wild-type plants (Veljovic-Jovanovic et al. 2001).

During flower development, characteristic changes in ROS levels suggest that various ROS may have specific functions during flowering and AsA/DHA redox pairs are mainly involved in modulating the required redox homoeostasis (Zafra et al. 2010). Analysis of AsA-deficient *Arabidopsis* mutants *vtc1-1*, *vtc2-1*, *vtc3-1*, and *vtc4-1* growing under short and long day length conditions has proposed that AsA may affect flowering time. Circadian clock and photoperiodic pathways genes were significantly higher in the *vtc* mutants, which exhibited an early flowering phenotype compared to wild type. Moreover, genetic analysis demonstrated that periodic and autonomous pathway mutants were epistatic to the *vtc1-1* mutant (Kotchoni et al. 2009). This conclusion could be supported by the study in *Oncidium* orchid, where a decreased AsA redox state acts as a signal to initiate flowering (Chin et al. 2016).

3 The Role of AsA/DHA Redox Pair Under Abiotic and Biotic Stress Responses

A plant subjected to stress will suffer a series of strains, the intensity of each strain will be directly proportional to the magnitude and duration of the stressor and inversely proportional to the strain tolerance of the plant (Blum 2016; Levitt 1972). Each plant might present constitutive resistance. Besides, the strain itself and/or physiological effects caused by the strain may represent a signal perceived by the plant to promote an adaptive resistance (Blum 2016). For many different stresses, there are mechanisms of avoidance, tolerance, and perception that rely in the physicochemical properties of the redox pair AsA/DHA and its central role in plants metabolism and signalling together with the glutathione redox pair (Gest et al. 2013; Noctor 2006). Generally, plants with low AsA biosynthesis or AsA recycling capacity are more sensitive to environmental stressors. Besides, increases in DHAR activity that represent changes in AsA redox state but not pool size cause enhanced tolerance to many stress: low temperature, salinity, toxic metals, methyl viologen (generating $O_2^{\bullet -}$), or H_2O_2 (Gallie 2013; Kwon et al. 2003). In the next sections, tolerance responses to different stresses in which ascorbate and its redox state play a role will be presented and summarized in Table 1.

Table 1 Summary of the discussed environmental stresses and the corresponding strains generated in the plant

Stress	Strain	Avoidance/adaptation mechanisms	Molecular mechanisms	References
High light	Photo-oxidative stress	Excess energy dissipation. ROS and oxidative damage detoxification	Water–water cycle and ascorbate as alternative acceptor/donor in chloroplasts, xanthophylls cycle, ROS and oxidative damage scavenge, antioxidants regeneration	Asada (2006), Demmig-Adams et al. (2012), Kozuleva et al. (2016), Mano et al. (2004), Murata et al. (2012), Tóth et al. (2011)
High/low temperature	Metabolism disruption. Photo-oxidative stress	Excess energy dissipation. ROS and oxidative damage detoxification	Water–water cycle and ascorbate as alternative acceptor/donor in chloroplasts and mitochondria; xanthophylls cycle; ROS, RNS and oxidative damage scavenge, antioxidants regeneration; ascorbate synthesis modulation	Asada (2006), Demmig-Adams et al. (2012), Mano et al. (2004), Bita and Gerats (2013), Lázaro et al. (2013), Martí et al. (2011), Míguez et al. (2015), Szarka et al. (2004, 2013), Tóth et al. (2011)
	Lipid peroxidation	Oxidative damage detoxification	Oxidative damage scavenge, antioxidants regeneration (notably alpha-tocopherol)	Munné-Bosch(2005)
Water shortage	Osmotic strain	Stomatal regulation	Apoplastic oxidative burst modulation, redox signalling	Chen and Gallie (2004), Fotopoulos et al. (2008)
	Photo-oxidative stress	Excess energy dissipation. ROS and oxidative damage detoxification	Water–water cycle and ascorbate as alternative acceptor/donor in chloroplasts; xanthophylls cycle; ROS and oxidative damage scavenge, antioxidants regeneration	Asada (2006), Demmig-Adams et al. (2012), Mano et al. (2004), Murata et al. (2012), Pastore et al. (2006), Schroeder et al. (2001), Tóth et al. (2011)
Water excess	Hypoxia/anoxia	Excess energy dissipation. ROS and oxidative damage detoxification. Respiratory metabolism modulation	Water–water cycle and ascorbate as alternative acceptor/donor in chloroplasts and mitochondria; xanthophylls cycle; ROS, RNS and oxidative damage scavenge, antioxidants regeneration; ascorbate synthesis modulation; DHA phloem transport	Asada (2006), Demmig-Adams et al. (2012), Franceschi (2002), Herschbach et al. (2010), Mano et al. (2004), Murata et al. (2012), Tóth et al. (2011)
Salinity	Osmotic strain	Stomatal regulation	Apoplastic oxidative burst modulation, redox signalling	Chen and Gallie (2004), Fotopoulos et al. (2008)
		Osmotic adjustment	Free amino acids modulation	Gulyás et al. (2017), Sharma and Dietz (2006), Vranova et al. (2011)

	Ion toxicity	ROS and oxidative damage detoxification	Water–water cycle and ascorbate as alternative acceptor/donor in chloroplasts and mitochondria; xanthophylls cycle; ROS, RNS and oxidative damage scavenge, antioxidants regeneration	Asada (2006), Demmig-Adams et al. (2012), Mano et al. (2004), Murata et al. (2012), Tóth et al. (2011)
Metal toxicity	Metabolism disruption. Oxidative stress and photo-oxidative stress	Excess energy dissipation. ROS and oxidative damage detoxification	Water–water cycle and ascorbate as alternative acceptor/donor in chloroplasts and mitochondria; xanthophylls cycle; ROS, RNS and oxidative damage scavenge, antioxidants regeneration	Asada (2006), Buettner and Jurkiewicz (1996), Demmig-Adams et al. (2012), Mano et al. (2004), Murata et al. (2012), Tóth et al. (2011)
		Chelation	Free amino acids modulation	Gulyás et al. (2017), Sharma and Dietz (2006), Vranova et al. (2011)
Environmental pollutants	Oxidative stress	Stomatal regulation	Oxidative burst modulation, redox signalling	Vainonen and Kangasjärvi (2015), Yoshida (2005)
		ROS and oxidative damage detoxification	Water–water cycle and ascorbate as alternative acceptor/donor in chloroplasts and mitochondria; ROS, RNS and oxidative damage scavenge, antioxidants regeneration	Asada (2006), Burkey et al. (2003), Demmig-Adams et al. (2012), Mano et al. (2004), Murata et al. (2012), Plöchl et al. (2000), Tóth et al. (2011)

Next to each strain, associated avoidance/adaptation mechanisms are summarized in which the ascorbate pool or it redox state plays a role in their known molecular mechanisms.

3.1 AsA Redox State in the Chloroplast Under High Light Stress

Sudden or long exposure to high light causes stress to the chloroplast functionality (Asada 2006). The main detrimental consequence of high irradiance is oxidative stress caused when absorbed light exceeds photosynthetic capacity, namely photo-oxidative stress. But other environmental stressors can generate photo-oxidative stress in the chloroplast without increased incident energy but impairing photosynthetic capacity and the linked metabolism (see Table 1).

Under excess light, two major ROS generating events occur in chloroplasts. An excess of excited chlorophyll (due to high irradiance and/or a disturbed photosynthetic metabolism) converts molecular oxygen (O_2) into singlet oxygen ($^1O_2$) (Krieger-Liszkay 2005). Downstream the electron transport chain, excess of reduced phylloquinone, ferredoxin and Fe-S clusters in the PSI reaction center complex relative to limiting electron and reducing power acceptors can use molecular oxygen as an alternative electron acceptor to generate superoxide anion (O_2^-), a highly reactive species that rapidly reacts to generate oxidative damage and/or other ROS (Asada 2006). Under optimal conditions, these ROS generating reactions occur at low rates because light energy transferred to excited chlorophylls is efficiently used to power PSI and PSII and ferredoxin readily reduces NADP+ in PSI as final electron acceptor. ROS will be formed at increased rates only under excess light because of (1) a limitation in the capacity of the plant to consume NADPH in chloroplasts due to environmental stressors (e.g., drought induced stomatal closure limiting Calvin cycle NADPH consumption, anoxia, temperature stress), (2) high irradiance (e.g., midday conditions in a sunny day), and/or (3) environmental stressors disrupt the chloroplast electron transport chain itself and/or its repair mechanisms (e.g., high temperature denaturing electron transport chain proteins or toxic metal ions disrupting the electron transport). Thus, several environmental stressors such as drought, salinity, nutrient deficiency, high and low temperature, or environmental pollutants lead to increased ROS production in chloroplasts (Demidchik 2015; Kozuleva et al. 2016; Murata et al. 2012). Excessive absorbed energy is dissipated by a number of mechanisms to limit photodamage: LHCII state transitions, xanthophyll cycle, photorespiration, ROS production and detoxification mechanisms (such as the water–water cycle) (Demmig-Adams et al. 2012; Li et al. 2009). Notably, the AsA pool and its redox state play an essential role in most of these mechanisms.

AsA is necessary for the xanthophyll cycle. In this mechanism of dissipation of excessive excitation energy in the PSI, the enzyme violaxanthin de-epoxidase uses excess energy to convert the carotenoid violaxanthin to zeaxanthin, requiring a readily available pool of reduced ascorbate as cosubstrate (Eskling et al. 1997; Yamamoto et al. 1962). Meanwhile, photorespiration reactions dissipate excess reducing power and energy directly by using ATP, NAD(P)H, and reduced ferredoxin (Peterhansel et al. 2010; Wingler et al. 2000). At the same time, the required ribulose-1,5-bisphosphate regeneration generates H_2O_2 in the peroxisome, possibly involved in signal transduction and redox homeostasis adjustment (Scheibe and

Dietz 2012; Takahashi et al. 2007; Voss et al. 2013). AsA contributes to H_2O_2 detoxification in the peroxisome as cosubstrate of APX (Karyotou and Donaldson 2005). Thus, the maintenance of AsA redox state and pool in the peroxisome is essential for the detoxification and fine-tuning of the redox homeostasis feedback signal generated by photorespiration metabolites.

The above described mechanisms dependent of the AsA pool and its redox state reduce the probability of ROS production. Once ROS generation rises, the availability of reduced ascorbate and readily available recycling mechanisms can maintain minimal ROS steady levels through the water–water cycle (Asada 1999, 2006). This cycle consists in the photoreduction of molecular oxygen to superoxide in PSI by electrons generated in PSII from water, while half of the electrons are used to regenerate ascorbate. The generated superoxide can generate oxidative damage to nearby macromolecules, but is readily transformed in H_2O_2 by superoxide dismutases. The regenerated AsA provides reduced equivalents to detoxify H_2O_2 to water by ascorbate peroxidase (APX), and detoxify products from oxidative damage (Asada 1999, 2006). This constitutes a nonproductive cycle that provides ROS scavenging and protection from photoinhibition, in which readily available reduced AsA is essential. In addition, AsA can nonenzymatically detoxify ROS, such as $^1O_2$ (Chou and Khan 1983) and $OH^{\bullet}$ (Buettner and Jurkiewicz 1996), as well as recycle tocopheroxyl radicals to tocopherol, generated by the scavenging of lipid peroxyl radicals caused by oxidative damage on lipids (Munné-Bosch 2005). In addition, different APX isoforms reduce H_2O_2 using AsA as reducing power in other plant cell compartments (Pandey et al. 2017). Thus, a readily reduced AsA pool and recycling mechanisms are essential to sustain these detoxification mechanisms for the plant survival.

When the photosynthetic electron transport chain is impaired, reduced and oxidized AsA forms can act as an alternative donor and acceptor of electrons in order to avoid photo-oxidative damage. If the primary electron donor system is impaired, AsA can be a direct electron donor to PSI and PSII generating MDHA in the lumen (Mano et al. 1997, 2004). Hence, AsA enables cyclic electron transport in anoxic conditions, high temperature, or when the electron transport chain reaction centers are impaired under severe nutritional deficits (Tóth et al. 2009, 2013). Meanwhile, when the electron acceptor $NADP^+$ becomes limiting, photoreduction of MDHA to AsA by ferredoxin can maintain electron transport (Forti and Ehrenheim 1993; Miyake and Asada 1994) protecting the photosynthetic machinery by slowing down photo-oxidative damage (Tóth et al. 2011). In addition, it has recently been described that light acclimation processes and the resulting photosynthesis rate are influenced not only by chloroplastic AsA redox state, but also by the apoplastic redox state (Karpinska et al. in press), largely regulated by AO activity (discussed in latter sections).

Accumulated DHA can conjugate with peptides and proteins cysteine thiol groups to generate DHA-peptide or DHA-protein adducts (Kay et al. 2013). These modifications might be generalized while at the same time specific to certain peptides and proteins (i.e., glutathione, glutaredoxin) in oxidative stress-mediated cellular toxicity (Flandrin et al. 2015; Regulus et al. 2010). The relevance of this

modification mechanism for oxidative stress perception and response in vivo in plants and other organisms remains to be explored.

As discussed elsewhere, AsA has plenty of functions as cofactor or cosubstrate of many enzymatic reactions (Arrigoni and De Tullio 2002). But DHA also plays a role as an oxidant in the protein-disulfide isomerase-catalyzed protein disulfide formation, essential for oxidative protein folding in the endoplasmatic reticulum (Bánhegyi et al. 2003; Csala et al. 1999; Nardai et al. 2001), an essential step of the secretory protein pathway. The established disulfides can act as thiol switches for regulatory processes like short-term adaptation to normal daily environmental changes such as dark–light cycle, but also to sense oxidative challenges during severe environmental stresses (Onda 2013).

The photosynthetic electron transport can be strained by an imbalance between incident energy and electron flux capacity caused by many environmental stressors, potentially generating ROS and photo-oxidative damage. The size of the ascorbate pool and its redox state are essential in a number of energy dissipation mechanisms associated to photo-oxidative stress, preventing the over-reduction of the photosynthetic electron chain and ultimately avoiding photo-oxidative damage. All these energy dissipation mechanisms require a readily available pool of reduced AsA in different compartments and AsA recycling mechanisms. The potential regulatory role in stress signalling and responses of DHA adducts and DHA in oxidative protein folding remain to be examined.

3.2 AsA Redox State in the Mitochondria Under Temperature Stress

Temperature stress is one of the main environmental limitations affecting plant production. Extreme temperatures affect plants by three strains: lowering enzymatic activity—by thermodynamic limitations or loss of conformation in low or high temperature, respectively—; loss of membrane function, by changes in membrane fluidity and lipid peroxidation promotion; and ice formation and mechanical damage (Bita and Gerats 2013; Thomashow 1999).

High or low temperature exposure results in direct disruptions in proteins and membranes due to temperature itself, and indirectly because of the subsequent increased ROS generation. Therefore chloroplast and mitochondrial metabolism can be severely disturbed, being energy dissipation and ROS detoxification mechanisms essential in the response to temperature stress (Iba 2002; Larkindale and Knight 2002; Míguez et al. 2015). In previous sections, it has been already discussed how the disturbance of the chloroplastic electron chain by excessive absorbed energy is determined by ascorbate content due to its redox state and its double role as an alternative electron donor and acceptor, as well as its involvement in ROS detoxification mechanisms. These mechanisms are also relevant in temperature stress adaptation, where enzymes and membranes functionality can be compromised (Bita and Gerats

2013). Thus, the ascorbate pool and redox state are essential for minimizing the damage to photosynthetic apparatus caused by temperatures stress.

Although the mitochondrial ROS production is considerably less than in illuminated chloroplasts or in peroxisomes, in most conditions, but especially in the dark or in non-green tissues, mitochondria are major generators of ROS (Lázaro et al. 2013; Noctor and Foyer 2016). The DHA transporter located in the inner mitochondrial membrane is an important member of the ascorbate regeneration machinery (Szarka et al. 2004). Similarly to the mechanisms described to prevent excessive energy in the chloroplastic electron chain, DHA can be an alternative acceptor in the mitochondrial complex II while ascorbic acid can act as an electron donor to complex IV (Szarka et al. 2007, 2013). This electron route through the AsA/DHA pair may provide an alternative route in case of complex III damage by taking up electrons at complex II (by the reduction of DHA to ascorbate) and providing electrons through a bypass to complex IV (by the oxidation of ascorbate to DHA) (Szarka et al. 2013). Plant mitochondria have also emerged as an important site for reactive nitrogen species (RNS), and these reactive species, like ROS, can generate oxidative damage but also present signalling roles generating reversible changes in the redox state of target molecules. In addition, RNS are involved in S-glutathionylation and S-nitrosylation protein modifications (Lázaro et al. 2013). These functions are important in response to stress conditions and are actively modulated by antioxidant mechanisms like the ascorbate-glutathione cycle (Martí et al. 2011).

It should be reminded that the last step of AsA biosynthesis is dependent on respiratory electrons, and therefore the respiratory electron flow (and thus the respiratory rate) has a regulatory role in ascorbate biosynthesis (Millar et al. 2003). In mitochondria, both AsA synthesis and recycling depend on respiratory electrons, thus perturbations of mitochondrial metabolism will cause respiration-dependent changes of AsA metabolism that would regulate retrograde signalling as a common signal from both mitochondria and chloroplasts (Szarka et al. 2007; Talla et al. 2011). An example of such an inter-organelle communication is the AsA produced in the mitochondria and then transported into the apoplast. Thus, AsA levels and redox state have been shown to modulate photosynthesis through mitochondrial metabolism in order to protect photosynthesis against photoinhibition (Lázaro et al. 2013; Talla et al. 2011), especially under heat, salinity, and drought stress (Lázaro et al. 2013; Pastore et al. 2006).

Extreme temperatures can impose severe disturbances to electron transport chains in chloroplast and mitochondria, requiring excess energy dissipation mechanisms, as well as ROS and oxidative damage detoxification mechanisms where ascorbate pool and redox state play an essential role. As AsA is synthesized and recycled in mitochondria with electrons from the respiratory electron chain and is sensible to its electron flux, there is a possible role for ascorbate in coordinating the rates of respiration, the tricarboxylic acid cycle, and photosynthesis to environmental and developmental stressors.

3.3 Drought and Salt Stress

The stomatal closure caused by drought and subsequent limitation of CO_2 entry reduces NADPH consumption for assimilation, and thus the available $NADP^+$. When this final electron acceptor is limited, the chloroplastic electron chain can accumulate excess energy, dissipated by the above described mechanisms that require a readily available ascorbate pool and redox state (see Table 1 and Sect. 3.1). Nevertheless, these are not the only tolerance mechanisms in which AsA pool and redox state are essentials.

In the apoplast, ROS are not only damaging reactive species, but also signalling molecules, like H_2O_2 in guard cells which controls gas exchange. Absorbed light energy excess, for example during peak sunlight when irradiance can exceed photosystem's photosynthetic capacity, can increase H_2O_2 production, triggering stomatal closure (Schroeder et al. 2001). During the day, AsA is consumed for H_2O_2 reduction and the rate-limiting DHAR activity causes an increase of DHA contents. Thus, the signalling role of H_2O_2 in stomatal conductance is modulated by both its rate of productions and removal, the latter determined by the AsA pools, its redox state, and DHAR recycling activity. The manipulation of DHAR activity, either overexpressed or suppressed, changes the efficiency of AsA recycling and thus H_2O_2 scavenging. DHAR overexpression increases AsA/DHA redox state towards a more efficient H_2O_2 scavenging, maintaining H_2O_2 at levels that do not trigger stomatal closure, therefore presenting increased transpiration and water loss under both normal and water stress conditions (Chen et al. 2003; Chen and Gallie 2004). Meanwhile, the suppression of DHAR expression causes an accumulation of H_2O_2, inducing more stomatal closure under the same water conditions (Chen and Gallie 2004). Actually, exogenous DHA promotes rapid stomatal closure, while AO overexpressing plants present impaired control of stomatal aperture, suggesting that not only the redox state but also the DHA levels and its modulation are regulators of stomatal dynamics, a key trait of tolerance to drought (Fotopoulos et al. 2008).

The size and redox state of the AsA pool influences plant metabolism and its plasticity for stress tolerance responses not only through AsA antioxidant functions. For example, influencing free amino acid levels. The manipulation of levels of AsA, DHA and the AsA redox state by the combination of mutants and chemical treatments with AsA, reduced glutathione and the synthetic reductant dithiothreitol (DTT), showed their influence on total free amino acid levels, and individual proteinogenic and non-proteinogenic amino acids levels (Gulyás et al. 2017). The actual redox state of AsA (and GSH) regulates free amino acids levels because this regulation was not observed by the synthetic reductant DTT (Gulyás et al. 2017). Free amino acids are essential in response against stresses, specially salt, heavy metal, and drought stress where free amino acids and derived molecules contribute to osmotic adjustment, metal binding, antioxidant defense, and signalling (Sharma and Dietz 2006; Vranova et al. 2011).

The AsA redox state in different compartments can actively modulate tolerance responses like stomatal closure and free amino acids contents, contributing to limiting transpiration, and promoting osmotic adjustments and metal chelation.

3.4 Flooding

Generation of reactive oxygen species (ROS) is characteristic for hypoxia and especially for reoxygenation. Consequences of hypoxia-induced oxidative stress depend on the tolerance to anoxia, membrane properties, endogenous antioxidant content, and on the ability to induce the response in the antioxidant systems. The switch to anaerobic metabolism and the preservation of the redox status are necessary for survival. Overexpressing experiments leading to increased antioxidants production do not always result in the enhancement of the antioxidant defense shield, and hence increased antioxidant capacity does not always correlate positively with the degree of protection (Herschbach et al. 2010). Plants tolerant to anaerobic conditions present significant increase in the reduced forms of ascorbate and ascorbate recycling enzymes, but if the stress endures recycling enzymes and AsA/DHA ratio decrease. DHA is the transported form of ascorbate through the phloem, generally from photosynthetic tissues to autotrophic organs and tissues with limited oxygen availability like root tips (Franceschi 2002; Herschbach et al. 2010). The interruption of this transport changes ascorbate contents and redox state across the entire root system (Herschbach et al. 2010). This transport might be important to act as a signal of redox imbalance due to stress between different tissues/organs, as shoot to root signal to coordinate growth and response to environmental stressors.

3.5 Metal Stress

Heavy metals generate a disturbance of the cellular redox balance and impair metabolism, leading to a rise of ROS generation. AsA pool and redox state play essential roles in avoidance (chelation) and tolerance mechanisms (e.g., ROS detoxification).

As discussed above, DHA content modulates free amino acids contents, notably many with chelating functionality. For example, hydroxyl radicals ($^{\bullet}OH$) generated by trace levels of transition metals by the metal-catalyzed Haber–Weiss reaction (producing $^{\bullet}OH$ from H_2O_2 and O_2^-) can nonenzymatically be detoxified by AsA (Buettner and Jurkiewicz 1996). Previously presented enzymatic and nonenzymatic ROS scavenging mechanisms that require a readily available pool of reduced AsA are also relevant in metal stress. Moreover, trace levels of transition metals can catalyze the oxidation of AsA (Buettner and Jurkiewicz 1996). Similarly, it has been observed that under high concentrations of iron together with high rates of AsA recycling leading to a sustained reduced AsA redox state, confer sensitivity to

exposed plants. This result suggests the existence of pro-oxidant activity of reduced AsA in the presence of high concentrations of iron *in planta* (Wu et al. 2017).

3.6 AsA Redox State in the Apoplast: Biotic Stress and Environmental Oxidative Pollutants

Ozone and other oxidative pollutants (notably nitrogen and sulfur oxides) rapidly degrade into hydroxyl when entering a plant through guard cells, rapidly spreading the oxidative strain to other cells (Sandermann et al. 1998). Damage extension is limited by avoidance, closing stomata to reduce their entrance into leaves; or by tolerance, detoxifying ROS that get into the plant (Vainonen and Kangasjärvi 2015). Apoplastic AsA acts directly as a first line of defense against environmental oxidants such as ozone, SO_2, and NO_2 (Plöchl et al. 2000), being ozone tolerance correlated to the levels of apoplastic AsA (Burkey et al. 2003). As described previously, AsA recycling capacity and apoplastic AsA redox state and DHA levels regulate the responsiveness of guard cells to ROS accumulation. Thus, apoplastic DHA levels and AsA redox state modulate stomatal closure as a pollutants avoidance mechanism (Yoshida 2005).

There is significant overlap between the signalling pathways and response factors activated by pathogens and those induced by ozone or reduced ascorbate contents (Bostock et al. 2014; Sandermann et al. 1998). Probably, common signalling pathways are triggered by increased apoplastic oxidative stress, as promoted by both environmental oxidants and the pathogen-induced oxidative burst during the hypersensitive response. AsA levels, AsA redox state, and AsA recycling capacity are strongly associated with resistance to pathogenic virus (Fujiwara et al. 2016), bacteria, and fungi (Botanga et al. 2012).

The AsA pool and metabolism in the apoplast regulates environmental perception and its signalling transduction. Environmental oxidative pollutants and pathogen attack oxidize the apoplast promoting a phenomenon named oxidative burst. As the major antioxidant buffer in the apoplastic compartment AsA modulates this signalling and it is associated to developmental and tolerance responses (Horemans et al. 2000; Pignocchi and Foyer 2003). Actually, AsA levels and especially AsA redox state are actively modulated by the apoplastic AO. A number of AO isoforms are differentially expressed in different organs, being actively regulated by both developmental cues and in response to stress conditions (Batth et al. 2017; Ioannidi et al. 2009). The deregulation of AsA redox state via AO overexpression enhances sensitivity to oxidative stress-promoting agents like ozone with an associated suppression of ascorbate recycling genes expression (Fotopoulos et al. 2006; Sanmartin et al. 2003). However, increased AO activity also increased sensitivity to fungal infection, although without showing suppression of AsA recycling genes expression (Fotopoulos et al. 2006). AsA redox state and its modulation through AO are essential for adequate responses to pathogens, becoming a target of virulence factors.

AO function can be actively disrupted by pathogens like the cucumber mosaic virus. In early infection, the movement protein of cucumber mosaic virus associates with apoplastic AO disrupting the formation of functional AO dimers, thus enhancing the spread of virus to nearby cells and reducing the redox defense of the plant during initial stages of infection (Kumari et al. 2016). Open stomata are the main access point of many pathogens, thus the promoted oxidative bursts lead to stomata closure, generating an avoidance response (Bostock et al. 2014). Stomatal closure dynamics are also modulated by AO activity (Fotopoulos et al. 2008).

Oxidized forms of AsA are essential for trans-plasma membrane electron transfer, through the family cytochromes b561 dual function as MDHAR and as Fe^{3+}-reductases (Asard et al. 2013; Griesen et al. 2004; Picco et al. 2015). It is also present in the vacuoles membrane where it may participate in storing excessive iron, but its biological functions are better described in the plasma membrane as essential for recycling AsA and for iron uptake. In addition, they act as buffering oxidant pollutants and modulating the signalling associated to the oxidative burst caused by pathogens.

Apoplastic AsA pool and redox state is actively modulated by apoplastic AO and plasmalemma cytochromes b561, and this modulation is essential for proper abiotic and biotic stress avoidance and tolerance responses. Mechanisms regulated by the AsA/DHA redox pair in the apoplast include signalling modulation (oxidative burst), stomatal closure, antioxidant buffer and environmental oxidants detoxification.

4 Conclusions and Perspectives

Ascorbate is the most abundant hydrosoluble antioxidant in plants, with multiple antioxidant and non-antioxidant functions. It is not only the major redox buffer with regulatory roles in many compartments, but it is also a cosubstrate of essential enzymatic reactions. The active modulation of ascorbate redox state in specific compartments is essential to successfully regulate development and growth while integrating environmental stimuli to modulate stress responses.

The modification of AsA/DHA relative contents by exogenous applications and molecular techniques has revealed many developmental and growth processes regulated by the AsA pool and redox state. However, our explanation of the underlying mechanisms still lacks specific targets or receptors of these disturbances. A highly active focus of ascorbate research is the regulation of the transport of the specific AsA, DHA and its redox power between compartments and between tissues, and its relevance in developmental and stress responses. The maintenance of specific AsA/DHA redox state in different compartments is essential to successfully regulate development and growth and to modulate avoidance and tolerance stress responses. Ascorbate is synthesized in the mitochondria but required for all compartments for

its detoxifying and regulatory roles, as well as a cosubstrate of enzymatic reactions and to coordinate plant energy metabolism. In addition to the plasma membrane DHA transporter (Horemans et al. 1997), other regulatory elements of the relationship of ascorbate contents and its redox state between compartments have only recently been described, and the study of their functional regulation is an active field. A recent study characterized the chloroplast transporter AtPHT4;4 while providing indications of a number of other possible plastidial ascorbate transporters (Miyaji et al. 2015). Yet many putative transporters remain to be identified and characterized, necessary to unravel the dynamics between different compartments. In addition, the recently described cytochrome b561 capacity to use ascorbate reduction power across compartments (Picco et al. 2015) raises many questions about the relationship between ascorbate pools and redox states in different compartments, its regulation, and its potential regulation role.

Despite plant cell energetic metabolism is essential for adaptation to environmental and developmental stressors; mitochondrial metabolism under stress is still largely unknown but there are hint that AsA and its redox state participate in its adaptation and coordination with other organelles. Both AsA and DHA participate in energy dissipation mechanisms in the electron transport chains of mitochondria and chloroplasts, while modulating ROS and RNS detoxification and related redox sensing. Therefore, the AsA pool and redox state may have a signalling role coordinating the chloroplast/cytosol/mitochondrion cooperation, especially under stress conditions, aimed at modulating cell redox homeostasis across all intra- and extracellular compartments.

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